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INTRODUCTION

A large body of research has focused on the pathological significance of calcium accumulation in the central nervous system (CNS) following cerebral ischemia, spinal cord injury and traumatic brain injury (TBI). Disturbances in neuronal calcium homeostasis may result in activation of several calcium sensitive enzymes including lipases, kinases, phosphotases and proteases. Recent studies have provided substantial evidence that activation of the calpains, calcium activated intracellular proteases, is a major pathological event in a number of acute CNS insults including TBI. Similarly, recent date have implicated another important family of cysteine proteases, caspases – especially caspase-3.

Much of the evidence on calpain and caspase activation following TBI has been indirect. The current project incorporates an integrated approach to studying protease activity in a clinically relevant rodent model of cortical impact injury using complementary techniques. Western blot immunoassays of calpain and caspae-3 specific BDP's proposed in SOW1 will be an efficient means of determining which brain regions and time points may be most usefully incorporated into studies in SOW2 providing more detailed information on mechanisms of calpain activation. Once we have a thorough understanding of calpain activity following TBI, we will initiate studies of calpastatin in SOW3. SOW4 will then investigate predictive relationships between changes in calpain and caspase activity and features of morphopathology produced by TBI. Studies will initially focus on five brain regions (cortex, dorsal hippocampus, thalamus, striatum and corpus callosum) taken from zones ipsilateral and contralateral to the site of cortical impact injury.

BODY

Record Of Research Findings and Approaches

During the past year, the laboratory has made substantial progress in addressing the research goals of this proposal. An important goal of SOW 1 is to test the hypothesis that TBI produces early and sustained increases in calpain and caspase-3 activity, inferred by increases in calpain and caspase-3 specific breakdown products (SBDPs). We have recently completed studies of the accumulation of αII-spectrin and αII-SBDPs in cerebrospinal fluid after traumatic brain injury (TBI) [Pike et al., 2001-included with this report]. Following a moderate level (2.0 mm) of controlled cortical impact TBI in rodents, native αII-spectrin protein was decreased in brain tissue and increased in CSF from 24 h to 72 h after injury. In addition, calpain-specific SBDPs were observed to increase in both brain and CSF after injury. Increases in the calpain-specific 145 kDa SBDP in CSF were 244%, 530% and 665% of sham-injured control animals at 24 h, 48 h and 72 h after TBI, respectively. The caspae-3-specific SBDP was observed to increase in CSF in some animals and to a lesser degree. Importantly, levels of these proteins were undetectable in CSF of uninjured control rats. These results indicate that detection of αII-spectrin and αII-SBDPs is a powerful discriminator of outcome and protease activation after TBI. In accord with our previous studies, results also indicate that calpain may be a ore important effector of cell death after moderate TBI than caspase-3.

Importantly, we have also been able to make important progress testing the relative roles of calpain vs. caspase-3 proteases, another important component of SOW 1. Importantly, these novel studies incorporated concurrent assessments of caspase-8, a caspase that could potentially regulate activation of caspase-3 following experimental TBI (Beer et al., J Neurochem, 2001-

included with this report). This study investigated the temporal expression and cell subtype distribution of procaspase 8 and cleaved caspase 8 p20 from 1 h to 14 days after cortical impact-induced TBI in rats. Caspase 8 messenger RNA levels, estimated by semi-quantitative RT-PCR, were elevated from 1 h to 72 h in the traumatized cortex. Western blotting revealed increased immunoreactivity for procaspase 8 and the proteolytically active subunit of caspase 8, p20, in the ipsilateral cortex from 6 to 72 h after injury, with a peak at 24 h after TBI. Similar to our previous studies, immunoreactivity for the p18 fragment of activated caspase 3 also increased in the current study from 6 to 72 h after TBI, but peaked at a later timepoint (48h) as compared with proteolyzed caspase 8 p20. Immunohistologic examinations revealed increased expression of caspase I in neurons, astrocytes and oligodendrocytes. Assessment of DNA damage using TUNEL identified caspase 8- and caspase3- immunopositive cells with apoptotic-like morphology in the cortex ipsilateral to the injury site, and immunohistochemical investigations of caspase 8 and activated caspase 3 revealed expression of both proteases in cortical layers 2-5 after TBI. Quantitative analysis revealed that the number of caspase 8 positive cells exceeds the number of caspase 3 expressing cells up to 24 h after impact injury. In contrast, no evidence of caspase 8 and caspase 3 activation was seen in the ipsilateral hippocampus, contralateral cortex and hippocampus up to 14 days after the impact. Our results provide the first evidence of caspase 8 activation after experimental TBI and suggest that this may occur in neurons, astrocytes and oligodendrocytes. Our findings also suggest a contributory role of caspase 8 activation to caspase 3 mediated apoptotic cell death after experimental TBI *in vivo*.

In addition, we have initiated a number of real time PCR studies systematically examining the role of calpain isoforms, calpastatin and various caspases following TBI. We anticipate successful completion of these studies during the upcoming year.

Results

Calpain-specific α-spectrin fragmentation (145 kDa) was detected at the earliest time-point assayed (3h) in all brain regions except striatum after 1.6 or 2.0 mm TBI. The 145 kDa fragment was no longer detectable in cortex and hippocampus by 14 days post-injury. Interestingly, the 145 kDa fragment was still detectable in thalamus after 1.6 mm, but not 2.0 mm injury at 28 days post-injury. The pattern of calpain activation closely paralleled the pattern of neuronal atrophy in these regions as assayed by H&E staining. Increases in the caspase-3 specific fragment to α-spectrin (120 kDa) was not detected at any post-injury time point (See Figure in Appendix).

Materials and Methods

Surgical preparation and controlled cortical impact brain injury: Forty adult male Sprague-Dawley rats were anesthetized with 4% isoflurane in a carrier gas of 70% N_2O/30% O_2, intubated and mechanically ventilated on 2% isoflurane in 70% N_2O/30% O_2. Core body temperature was monitored continuously by a rectal thermistor probe and maintained at 36.5-37.5 degrees C. A unilateral craniotomy (7 mm diameter) was performed over the right cortex adjacent to the sagittal suture, midway between bregma and lambda. Care was taken to avoid penetrating the underlying dura matter.

Trauma was produced by impacting the exposed right cortex with a 6 mm diameter aluminum tip at a velocity of 4 m/s with a 2.3 mm compression. Sham-injured animals

underwent identical surgical procedures but were not subjected to impact injury. Following injury or sham-injury, animals were weaned from the ventilator and extubated. Appropriate pre- and post-injury management was maintained to insure that all guidelines set forth by the Guide for the Care and Use of Laboratory Animals were complied with.

Immunoblot analysis: Ten groups (n = 4 per group) of rats wee used in this study. Groups included sham-injured animals and traumatized animals sacrificed at post-injury time points of 15 min., 3, 6, 24, 48 and 72 hours and 7 and 14 days. At appropriate post-injury time points, animals were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and decapitated at the loss of the toe-pinch reflex. Following decapitation, ipsilateral and contralateral (to the site of impact) cortex, hippocampus, thalamus, and striatum were rapidly dissected, quickly frozen in liquid N_2, and stored at –80 degrees C.

Brain tissue was homogenized in ice cold homogenization buffer (20 mM PIPES pH 7.6, 2mM EGTA, 1mM EDTA, 1mM DTT, 0.5 mM PMSF, 50 μg/ml each of AEBSF, aprotinin, pepstatin., TLCK and TPCK). Protein concentrations were determined by bicinchoninic acid micro protein assays (Pierce, Inc., Rockford, IL) with albumin standards. Protein-balanced samples were prepared for polyacrylamide gel electrophoresis (PAGE) in a two-fold loading buffer (0.25 M Tris pH 6/8, 0.2 M DTT, 8% SDS, 0.02% bromophenol blue, and 20% glycerol in distilled H_20), heated for 10 min at 95 degrees C, and centrifuged for 1 min at 10,000 r.p.m. Thirty micrograms of each sample was resolved in a vertical electrophoresis chamber using 4% acrylamide stacking gel over a 7% acrylamide resolving gel for 1 hour at 200 V. For immunoblotting, separated proteins were laterally transferred to nitro-cellulose membranes using a transfer buffer consisting of 0.192 M glycine and 0.025 M Tris (pH 8.3) with 10% methanol at a constant voltage of 100 V for 1 hour at 4 degrees C. Blots were blocked overnight in 5% non-fat milk in 20 mM Tris-HCI, 0.15 M NaCl, and 0.005% Tween-20 at 4 degrees C. Comossie blue and Panceau red (Sigma, St. Louis, MO) were used to stain gels and nitrocellulose membranes (respectively) to confirm that equal amounts of protein were loaded in each lane.

Antibodies and immunolabeling: Immunoblots were probed with an anti-spectrin monoclonal antibody (clone AA6; catalog # FG 6090; Affiniti Research Products Ltd., UK) that detects intact non erythroid α-spectrin (240 kDa) and 150, 145 and 120 kDa α-spectrin breakdown products (SBDPs). The 150 kDa SBDP has been reported to be an α-spectrin cleavage product of calpains and caspase-3-like proteases. However, the 145 kDa SBDP is a specific proteolytic fragment of calpains, whereas the 120 kDa SBDP is a specific proteolytic fragment generated by caspase-3 (CPP32) activation. Following incubation with the primary antibody (1:6000 dilution) for 2 hours at room temperature, nitrocellulose membranes were incubated with a secondary antibody linked to horseradish peroxidase (dilution 1:20 000) for 1 hour. Enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL) reagents were used to visualize immunolabeling on high performance chemiluminescence film (Hyperfilm-ECL; Amersham International, UK).

KEY RESEARCH ACCOMPLISHMENTS

- Completion and publication of characterization of calpain-cleaved α-spectrin breakdown products in cerebrospinal fluid after TBI in rats.

- Completion and publication of a study of the temporal and spatial profile of caspase-8 and caspase-3 expression in proteolysis after experimental TBI in rats.

- Initiation of real time-PCR experiments examining calpain isoforms, calpastatin, and a variety of caspase-related proteins following experimental TBI.

REPORTABLE OUTCOMES (Included with report)

Brian R Pike, Jeremy Flint, Satavisha Dutta, Erik Johnson, Kevin K W Wang, and Ronald L Hayes. Accumulation of non-erythroid αII-spectrin and calpain-cleaved αII-spectrin breakdown products in cerebrospinal fluid after traumatic brain injury in rats. J Neurochemistry, 78, 1-11, 2001.

Ronny Beer, Gerhard Franz, Stanislaw Krajewski, Brian R Pike, Ronald L Hayes, John C Reed, Kevin K Wang, Christian Klimmer, Erich Schmutzhard, Werner Poewe and Andreas Kampfl. Temporal and spatial profile of caspase 8 expression and proteolysis after experimental traumatic brain injury. J of Neurochemistry, 78, 862-873, 2001.

CONCLUSIONS

The research within the second year has provided novel and important information indicating that calpain activation can be detected following TBI in the cerebrospinal fluid of injured animals. These data have important implications for clinical and laboratory studies of pathophysiological responses to TBI. It is possible that similar techniques can be used in humans to assess the presence of destructive proteases following TBI. If such is the case, this could lay the ground work for important and novel biochemical markers of injury following human TBI. More importantly, such markers would provide information not only in the magnitude of injury but the mechanisms underlying such injury.

Next, our studies have indicated that caspase-3 activity may play a more important role in experimental TBI than we had originally speculated. Equally important, our data suggests that regulation of caspase-3 activity may be via a precursor caspase, caspase-8. The knowledge of the regulation of caspase-3 activity following TBI could provide important insights into therapeutic opportunities to treat TBI.

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Journal of Neurochemistry, 2001, 78, 1–11

Accumulation of non-erythroid αII-spectrin and calpain-cleaved αII-spectrin breakdown products in cerebrospinal fluid after traumatic brain injury in rats

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Abstract

Although a number of increased CSF proteins have been correlated with brain damage and outcome after traumatic brain injury (TBI), a major limitation of currently tested biomarkers is a lack of specificity for defining neuropathological cascades. Identification of surrogate biomarkers that are elevated in CSF in response to brain injury and that offer insight into one or more pathological neurochemical events will provide critical information for appropriate administration of therapeutic compounds for treatment of TBI patients. Non-erythroid αII-spectrin is a cytoskeletal protein that is a substrate of both calpain and caspase-3 cysteine proteases. As we have previously demonstrated, cleavage of αII-spectrin by calpain and caspase-3 results in accumulation of protease-specific spectrin breakdown products (SBDPs) that can be used to monitor the magnitude and temporal duration of protease activation. However, accumulation of αII-spectrin and αII-SBDPs in CSF after TBI has never been examined. Following a moderate level (2.0 mm) of controlled cortical impact TBI in rodents, native αII-spectrin protein was decreased in brain tissue and increased in CSF from 24 h to 72 h after injury. In addition, calpain-specific SBDPs were observed to increase in both brain and CSF after injury. Increases in the calpain-specific 145 kDa SBDP were 237%, 203% and 198% of sham-injured control animals at 24 h, 48 h and 72 h after TBI, respectively. The caspase-3-specific SBDP was observed to increase in CSF in some animals and to a lesser degree. Importantly, levels of these proteins were undetectable in CSF of uninjured control rats. These results indicate that detection of αII-spectrin and αII-SBDPs is a powerful discriminator of outcome and protease activation after TBI. In accord with our previous studies, results also indicate that calpain may be a more important effector of cell death after moderate TBI than caspase-3.

Keywords: calpain, caspase-3, cell death, cerebrospinal fluid, spectrin, traumatic brain injury.

J. Neurochem. (2001) 78, 1–11.

The incidence of traumatic brain injury (TBI) in the United States of America is conservatively estimated to be more than 2 million persons annually with approximately 500 000 hospitalizations (Goldstein 1990). Of these, about 70 000–90 000 head injury survivors are permanently disabled. The annual economic cost to society for care of head-injured patients is estimated at $25 billion (Goldstein 1990). Thus, accurate and reliable measurement of outcome following head injury is of great interest to both head injury survivors and clinicians. Assessment of pathology and neurological impairment immediately after TBI is crucial for determination of appropriate clinical management and for predicting long-term outcome. The outcome measures most often used in head-injured patients are the Glasgow Coma Scale (GCS), the Glasgow Outcome Scale (GOS), and computed tomography (CT) scans to detect intracranial pathology.

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Abbreviations used: CT, computed tomography; GCS, Glasgow Coma Scale, GOS, Glasgow Outcome Scale, PVDF, polyvinylidene fluoride; SBDPs, spectrin breakdown products; TBI, traumatic brain injury.

However, despite dramatically improved emergency triage systems based on these outcome measures, most TBI survivors suffer long-term (for a number of years) impairment, and a large number of TBI survivors are severely affected by TBI despite predictions of 'good recovery' on the GOS (Marion 1996). Because of the limitations of current clinical assessments of TBI severity, there has been an increased interest in the development of neurochemical markers for determining injury severity and for clinical evaluation of pathophysiological mechanisms operative in traumatized brain.

For example, TBI results in neuronal tissue death that can cause a variety of neurochemicals such as amino acids, ions and lactate, as well as a number of cellular proteins and enzymes, to be released into the blood and CSF (Goodman and Simpson 1996). Although assessment of cardiac and liver protein levels in the blood has routinely been used in medical practice for years (e.g. creatine kinase MB or troponin-T), assessment of CNS proteins in blood or CSF is far less developed. Thus, recent studies have measured a variety of neurochemical substances in the CSF or blood in attempt to identify specific surrogate markers of cellular damage and outcome after TBI and other CNS disorders (Haber and Grossman 1980; Inao *et al.* 1988; Robinson *et al.* 1990; Lyeth *et al.* 1993; Raabe and Seifert 1999; Raabe *et al.* 1999; Zemlan *et al.* 1999; Clark *et al.* 2000a; Tapiola *et al.* 2000). For example, creatine kinase BB, lactate dehydrogenase, myelin basic protein, and neuron-specific enolase have been measured in blood or CSF in various CNS disorders including TBI. However, these proteins are non-specific to the brain, offer no insight as to mechanism of injury, and/or prediction of outcome utilizing these proteins has not proven reliable (Goodman and Simpson 1996). Other proteins detected in CSF after brain injury such as S-100B are highly specific to the CNS and have been more robustly correlated with the outcome (Raabe and Seifert 1999; Raabe *et al.* 1999). Although brain-specific surrogate biomarkers like S-100B may be useful indicators of outcome after brain injury, detection of these proteins in blood or CSF offers no insight into neurochemical alterations that mediate brain damage after TBI. Thus, identification of neurochemical markers that are specific to the CNS and that provide information about specific ongoing neurochemical events would prove immensely beneficial for both prediction of outcome and for guidance of targeted therapeutic delivery.

Non-erythroid αII-spectrin is the major structural component of the cortical membrane cytoskeleton, is particularly abundant in axons and presynaptic terminals (Riederer *et al.* 1986; Goodman *et al.* 1995), and is a major substrate for both calpain and caspase-3 cysteine proteases (Wang *et al.* 1998). The calpain-mediated cleavage of αII-spectrin occurs between Tyr^{1176} and Gly^{1177} resulting in the formation of calpain-signature spectrin breakdown products (SBDPs) of 150 and 145 kDa (Harris *et al.* 1988). The caspase-3-mediated cleavages of αII-spectrin occur at Asp^{1185}, Ser^{1186}, Asp^{1478} and Ser^{1479} resulting in the formation of caspase-3-signature SBDPs of 150 and 120 kDa, respectively (Wang *et al.* 1998). Importantly, numerous investigations have documented increased pathological activation of calpain and/or caspase-3 proteases after TBI (Saatman *et al.* 1996a, 2000; Kampfl *et al.* 1997; Newcomb *et al.* 1997; Posmantur *et al.* 1997; Yakovlev *et al.* 1997; Pike *et al.* 1998a; Clark *et al.* 1999, 2000b; LaPlaca *et al.* 1999; Okonkwo *et al.* 1999; Zhang *et al.* 1999; Beer *et al.* 2000; Buki *et al.* 2000). In addition, our laboratory and others have provided extensive evidence the αII-spectrin is processed by calpains and/or caspase-3 to signature cleavage products *in vivo* after TBI (Beer *et al.* 2000; Newcomb *et al.* 1997; Pike *et al.* 1998a; Buki *et al.* 2000) and in *in vitro* models of mechanical stretch injury (Pike *et al.* 2000b), necrosis (Zhao *et al.* 1999), apoptosis (Nath *et al.* 1996a, 1996b; Pike *et al.* 1998b), glutamate or NMDA excitotoxicity (Nath *et al.* 2000; Zhao *et al.* 2000), and oxygen-glucose deprivation (Nath *et al.* 1998; Newcomb *et al.* 1998). Moreover, use of selective αII-SBDP antibodies has been used to demonstrate that brain regions with the highest accumulation of SBDPs also have the highest levels of neuronal cell death (Roberts-Lewis *et al.* 1994; Newcomb *et al.* 1997). Thus, the ubiquitous distribution of αII-spectrin in the brain coupled with the ability to utilize signature αII-spectrin proteolytic fragments generated by pathological activation of calpain and/or caspase-3 after TBI makes αII-spectrin a potentially important biomarker of brain damage. To test this hypothesis, the present investigation examined alterations in brain levels of αII-spectrin and αII-SBDPs after controlled cortical impact TBI in rodents, and compared these changes to accumulation of αII-spectrin and αII-SBDPs in CSF in the same animals.

Materials and methods

Surgical Preparation and controlled cortical impact traumatic brain injury

As previously described (Dixon *et al.* 1991; Pike *et al.* 1998a), a cortical impact injury device was used to produce TBI in rodents. Cortical impact TBI results in cortical deformation within the vicinity of the impactor tip associated with contusion, and neuronal and axonal damage that is constrained in the hemisphere ipsilateral to the site of injury (Gennarelli 1994; Meaney *et al.* 1994). Adult male (280–300 *g*) Sprague-Dawley rats (Harlan; Indianapolis, IN, USA) were initially anesthetized with 4% isoflurane in a carrier gas of 1 : 1 O_2/N_2O (4 min) followed by maintenance anesthesia of 2.5% isoflurane in the same carrier gas. Core body temperature was monitored continuously by a rectal thermistor probe and maintained at 37 ± 1°C by placing an adjustable temperature controlled heating pad beneath the rats. Animals were mounted in a stereotactic frame in a prone position and secured by ear and

incisor bars. A midline cranial incision was made, the soft tissues were reflected, and a unilateral (ipsilateral to site of impact) craniotomy (7 mm diameter) was performed adjacent to the central suture, midway between bregma and lambda. The dura mater was kept intact over the cortex. Brain trauma in rats ($n = 9$) was produced by impacting the right cortex (ipsilateral cortex) with a 5-mm diameter aluminum impactor tip (housed in a pneumatic cylinder) at a velocity of 3.5 m/s with a 2.0-mm compression and 150 ms dwell time (compression duration). Velocity was controlled by adjusting the pressure (compressed N_2) supplied to the pneumatic cylinder. Velocity and dwell time were measured by a linear velocity displacment transducer (Lucas Shaevitz™ model 500 HR; Detroit, MI, USA) that produces an analogue signal that was recorded by a storage-trace oscilloscope (BK Precision, model 2522B; Placentia, CA, USA). Sham-injured animals ($n = 4$) underwent identical surgical procedures but did not receive an impact injury. Appropriate pre- and post-injury management was maintained to insure that all guidelines set forth by the University of Florida Institutional Animal Care and Use Committee and the National Institutes of Health guidelines detailed in the Guide for the Care and use of Laboratory Animals was complied with.

CSF and cortical tissue preparation

The CSF and brain cortices were collected from animals at various intervals after sham-injury or TBI. At the appropriate time-points, TBI or sham-injured animals were anesthetized as described above and secured in a stereotactic frame with the head allowed to move freely along the longitudinal axis. The head was flexed so that the external occipital protuberance in the neck was prominent and a dorsal midline incision was made over the cervical vertebrae and occiput. The atlanto-occipital membrane was exposed by blunt dissection and a 25G needle attached to polyethylene tubing was carefully lowered into the cisterna magna. Approximately 0.1–0.15 mL of CSF was collected from each rat. Following CSF collection, animals were removed from the stereotactic frame and immediately killed by decapitation. Ipsilateral and contralateral (to the impact site) cortices were then rapidly dissected, rinsed in ice cold PBS, and snap frozen in liquid nitrogen. Cortices beneath the craniotomies were excised to the level of the white matter and extended ~4 mm laterally and ~7 mm rostrocaudally. The CSF samples were centrifuged at 4000 *g* for 4 min at 4°C to clear any contaminating erythrocytes. Cleared CSF and frozen tissue samples were stored at −80°C until ready for use. Cortices were homogenized in a glass tube with a Teflon dounce pestle in 15 volumes of an ice-cold triple detergent lysis buffer (20 mM HEPES, 1 mM EDTA, 2 mM EGTA, 150 mM NaCl, 0.1% SDS, 1.0% IGEPAL 40, 0.5% deoxycholic acid, pH 7.5) containing a broad range protease inhibitor cocktail (cat. #1-836-145Roche Molecular Biochemicals, Indianapolis, IN, USA).

Immunoblot analyses of CSF and cortical tissues

Protein concentrations of tissue homogenates and CSF were determined by bicinchoninic acid microprotein assays (Pierce Inc., Rockford, IL, USA) with albumin standards. Protein balanced samples were prepared for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) in twofold loading buffer containing 0.25 M Tris (pH 6.8), 0.2 M DTT, 8% SDS, 0.02% bromophenol blue, and 20% glycerol in distilled H_2O. Samples were heated for 10 min at 100°C and centrifuged for 1 min at 10 000 r.p.m. in a microcentrifuge at ambient temperature. Forty micrograms of protein per lane was routinely resolved by SDS–PAGE on 6.5% Tris/glycine gels for 1 h at 200 V. Following electrophoresis, separated proteins were laterally transferred to polyvinylidene fluoride (PVDF) membranes in a transfer buffer containing 0.192 M glycine and 0.025 M Tris (pH 8.3) with 10% methanol at a constant voltage of 100 V for 1 h at 4°C. Blots were blocked for 1 h at ambient temperature in 5% non-fat milk in TBS and 0.05% Tween-20. Panceau Red (Sigma, St Louis, MO, USA) was used to stain membranes to confirm successful transfer of protein and to insure that an equal amount of protein was loaded in each lane.

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Antibodies and immunolabeling of PVDF membranes

Immunoblots containing brain or CSF protein were probed with an anti-α-spectrin (fodrin) monoclonal antibody (FG 6090 Ab; clone AA6; cat. # FG 6090; Affiniti Research Products Limited, UK) that detects intact non-erythroid αII-spectrin (280 kDa) and 150, 145 and 120 kDa cleavage fragments to αII-spectrin. A cleavage product of 150 kDa is initially produced by calpains or caspase-3 proteases (each proteolytic cleavage yields a unique amino-terminal region: Nath *et al.* 1996a; Wang *et al.* 1998). The calpain-generated 150 kDa product is further cleaved by calpain to yield a specific calpain signature product of 145 kDa (Harris *et al.* 1988; Nath *et al.* 1996a,b) whereas the caspase-3 generated 150 kDa product is further cleaved by caspase-3 to yield a specific caspase-3 signature product of 120 kDa (Nath *et al.* 1998; Wang *et al.* 1998). To further confirm the specificity of calpain-cleaved spectrin in CSF after TBI, a second antibody (anti-SBDP150; rabbit polyclonal) that recognizes only the calpain-cleaved N-terminal region (GMMPR) of the 150 kDa αII-spectrin breakdown product (SBDP) was also used (Saido *et al.* 1993; Nath *et al.* 1996b). Some immunoblots were immunolabeled with an antibody that recognizes erythroid αI-spectrin (Cat.# BYA10881; Accurate Chemical & Scientific Corp, Westbury, NY, USA). Following an overnight incubation at 4°C with the primary antibodies (FG 6090 Ab, 1 : 4000 for brain tissue and 1: 2000 for CSF; SBDP150 Ab, 1 : 1000; BYA10881, 1 : 400), blots were incubated for 1 h at ambient temperature in 3% non-fat milk that contained a horseradish peroxidase-conjugated goat anti-mouse IgG (1 : 10 000 dilution) or goat-anti-rabbit IgG (1 : 3000). Enhanced chemiluminescence (ECL; Amersham) reagents were used to visualize immunolabeling on Kodak Biomax ML chemiluminescent film.

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Statisitical analyses

Semi-quantitative evaluation of protein levels detected by immunblotting was performed by computer-assisted densitometric scanning (AlphaImager 2000; Digital Imaging System, San Leandro, CA, USA). Data were acquired as integrated densitometric values and transformed to percentages of the densitometric levels obtained on scans from sham-injured animals visualized on the same blot. Data was evaluated by least squares linear regression followed by ANOVA. All values are given as mean ± SEM. Differences were considered significant if $p < 0.05$.

Results

Proteolysis of αII-spectrin in the cortex by calpain, but not caspase-3 after TBI

In the ipsilateral cortex, TBI resulted in decreased protein levels of αII-spectrin (280 kDa) that were associated with concomitant accumulation of calpain-generated 150 and 145 kDa αII-SBDPs (Fig. 1). However, there was little to no detectable increase in the caspase-3-genearated 120 kDa αII-SBDP. These results replicate our previous investigation that reported calpain but not caspase-3 processing of αII-spectrin following a moderate level of lateral controlled cortical impact TBI (Pike *et al.* 1998a). Decreased αII-spectrin (280 kDa) protein levels were 65%, 48% and 39% of sham-injured protein levels at 24 h, 48 h, and 72 h after TBI, respectively (Fig. 2). Increased 150 kDa αII-SBDP levels were 189%, 157%, and 153% of sham-injured levels at 24 h, 48 h and 72 h after TBI, respectively, while increased 145 kDa αII-SBDP levels were 237%, 203% and 198% of sham-injured levels at 24 h, 48 h and 72 h after TBI, respectively (Fig. 2).

In the contralateral cortex, traumatic brain injury resulted in no apparent alteration in protein levels of αII-spectrin (280 kDa) or in any apparent accumulation of calpain-generated 150 or 145 kDa αII-SBDPs, or in caspase-3 generated 120 kDa αII-SBDP as compared to sham-injured control animals (Fig. 1). These results are also in accord with our previous report that calpain-mediated processing of αII-spectrin is predominately confined to ipsilateral brain regions after moderate lateral controlled cortical impact TBI (Newcomb *et al.* 1997; Pike *et al.* 1998a).

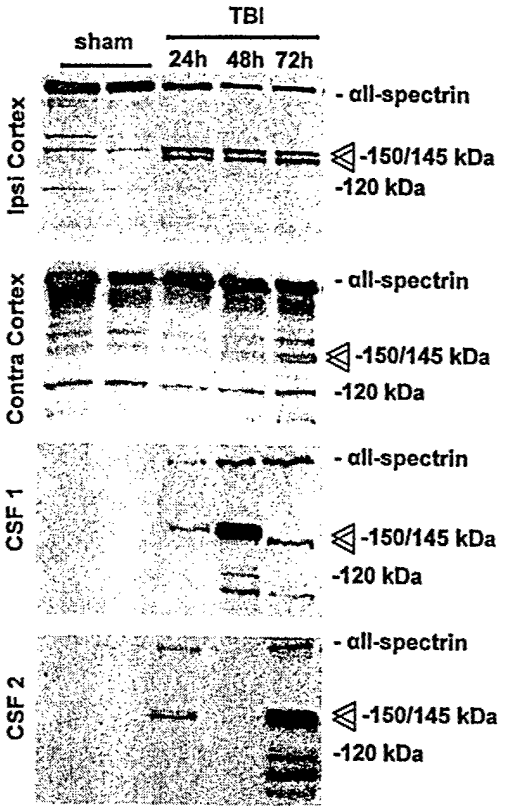

Fig. 1 Traumatic brain injury (TBI) results in prominent accumulation of αII-spectrin (280 kDa) and calpain-cleaved 150 kDa and 145 kDa αII-SBDPs in CSF (FG 6090 Ab). The caspase-3 generated 120 kDa fragment was also apparent in CSF of some animals. TBI caused proteolysis of constitutively expressed brain αII-spectrin (280 kDa) in ipsilateral but not contralateral cortex. Increases in the caspase-3-mediated 120 kDa αII-SBDP were not as apparent in ipsilateral or contralateral cortex after TBI. Immunolabeling of additional unknown bands at ~110 kDa and 95 kDa were also detected in CSF at 48 h and 72 h after injury. Both CSF1 and CSF2 are from two separate series of animals shown to illustrate that there was more variability in CSF levels of SBDPs than in brain levels, which may reflect individual differences in injury severity.

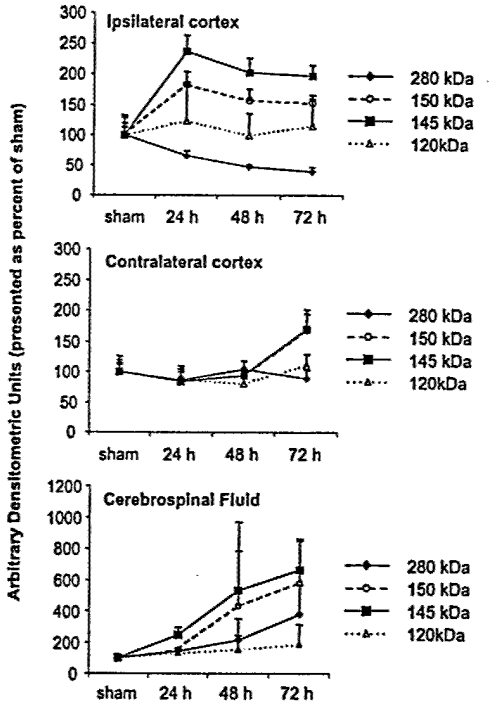

Fig. 2 Mean arbitrary densitometric units obtained from 280 kDa αII-spectrin and the 150 kDa and 145 kDa αII-SBDPs were converted to percent of sham-injured values. Decreases in 280 kDa αII-spectrin and increases in 150 kDa and 145 kDa αII-SBDPs (ipsilateral cortex) were associated with concomitant increases of these proteins in the CSF. Mean accumulation of the caspase-3 generated 120 kDa fragment in these tissues was relatively flat.

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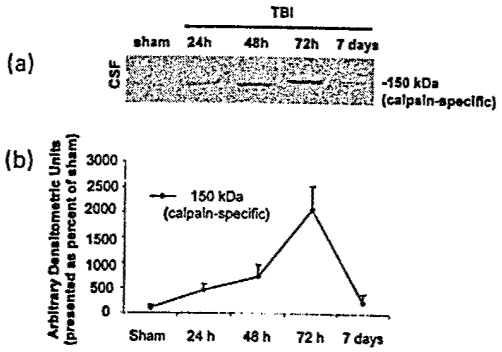

Fig. 3 N-terminal fragment-specific detection of calpain-generated SBDP150 in CSF after traumatic brain injury (SBDP150 Ab). (a) As with the FG 6090 Ab, the SBDP150 Ab detected a progressive increase in the calpain-cleaved 150 kDaA SBDP from 24 h to 72 h after TBI. Levels of 150 kDa SBDP had resolved to sham-injured control levels by seven days after TBI. (b) Mean arbitrary densitometric units of SBDP150 levels detected with anti-SBDP150 Ab.

Accumulation of calpain-mediated αII-SBDPs in CSF after TBI

Immunoblot analyses of CSF levels of non-erythroid αII-spectrin and αII-SBDPs (FG 6090 Ab) showed no detectable levels of these proteins in CSF of sham-injured control animals (Fig. 1). However, after TBI, accumulation of αII-spectrin (280 kDa) and calpain-generated 150 and 145 kDa SBDPs were markedly increased at 24 h, 48 h and 72 h, after injury (Fig. 1). In addition, there was an increase in the caspase-3-generated 120 kDa fragment in one animal at 48 h after TBI, and in another animal at 72 h after TBI (Fig. 1). Accumulation of αII-spectrin (280 kDa) protein levels was 143%, 212%, and 379% of sham-injured animals at 24 h, 48 h, and 72 h after TBI, respectively (Fig. 2). Similarly, accumulation of 150 kDa αII-SBDP after TBI was 155%, 434%, and 583% of sham-injured levels at 24 h, 48 h, and 72 h, respectively, while accumulation of 145 kDa αII-SBDP after TBI was 244%, 530%, and 665% of sham-injured levels at 24 h, 48 h and 72 h, respectively (Fig. 2). In contrast, although accumulation of the caspase-3 cleaved 120 kDa fragment was detected in two animals, the average response was relatively flat. In addition, several lower molecular weight species of αII-spectrin were detected. The protease(s) responsible for these lower molecular weight fragments are currently unknown. However, future identification of these bands may provide important new information regarding other neurochemical events in the brain after TBI.

To provide further confirmation of calpain-generated αII-SBDP accumulation in CSF after TBI, an additional group of animals ($n = 5$ per time-point) was injured as described above and immunoblots of CSF samples were probed with anti-SBDP150 Ab. In this experiment, an additional time-point (7 days post-TBI) was also examined. The SBDP150 Ab specifically recognizes only the calpain-cleaved 150 kDa αII-spectrin fragment and does not recognize the intact 280 kDa protein or other proteolytic fragments (Saido *et al.* 1993; Nath *et al.* (1996a). Results with the SBDP150 Ab were nearly identical to those obtained with the FG-6090 Ab (Fig. 3). The calpain-cleaved 150 kDa SBDP was nearly undetectable in CSF of sham-injured animals, and a progressive increase in immunoreactivity was observed from 24 h to 72 h after TBI. Importantly, this experiment also demonstrated that levels of calpain-cleaved 150 kDa SBDP were decreased back to sham-injured control levels by seven days after TBI (Fig. 3).

Q4 change to 'b'

Linear regression analyses of Cortical versus CSF levels of αII-spectrin and αII-SBDPs

Least squares linear regression was calculated to determine the relationship between brain and CSF levels of αII-spectrin and αII-SBDPs over days post-injury. The slope of the regression lines for αII-spectrin and αII-SBDPs in brain and CSF were analyzed by ANOVA.

For cortical levels of 280 kDa αII-spectrin protein, the slope of the regression line was relatively steep and negative indicating large decreases over days in cortical levels of native αII-spectrin protein (Fig. 4). In contrast, the slope of the regression line for CSF levels of 280 kDa αII-spectrin was relatively steep and positive indicating large increases over days in CSF levels of αII-spectrin protein after TBI. In addition, ANOVA indicated that there was a significant difference ($F = 19.95$, $p < 0.001$) between cortical and CSF slopes for 280 kDa αII-spectrin protein level. This significance indicates that as brain levels of αII-spectrin decrease over days, CSF levels of αII-spectrin increase over days.

For cortical and CSF levels of 150 kDa αII-SBDP, both slopes of the regressions lines were positive indicating large increases in the calpain-cleaved 150 kDa αII-SBDP in brain and CSF over days (Fig. 4). ANOVA indicated no significant difference ($F = 1.86$, $p = 0.1873$) between slopes indicating that the relative accumulation of 150 kDa αII-SBDP in cortex and CSF were similar. However, the slope for CSF 150 kDa αII-SBDP was relatively steeper than the slope for cortical 150 kDa αII-SBDP. This result reflects the densitometric data (Fig. 2) indicating that, in the cortex, peak levels of the 150 kDa αII-SBDP accumulated rapidly (24 h) and were maintained at 48 h and 72 h post-injury. This result also reflects densitometric data (Fig. 2) indicating that CSF levels of the 150 kDa αII-SBDP accumulated more slowly early after injury (24 h) with a greater rate of further accumulation at 48 h and 72 h post-injury. Observed statistical differences in accumulation rates can be appreciated visually in the immunoblot data (Fig. 1). The stability of αII-spectrin and αII-SBDPs in CSF may be increased due to lack of endogenous proteases. For example,

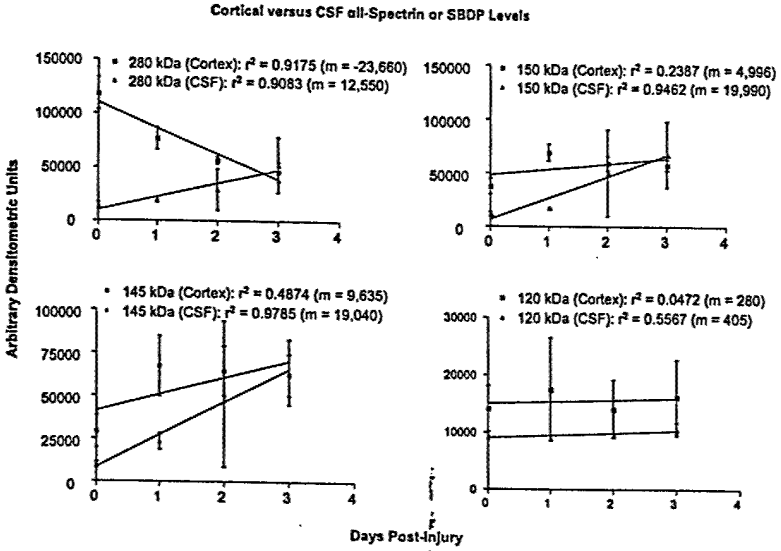

Fig. 4 Cortical versus CSF levels of αII-spectrin (280 kDa) and αII-SBDPs (150, 145, and 120 kDa) over days post-injury. Least squares regression lines of brain and CSF spectrin and SBDP levels were plotted on the same graph. Pearson correlation coefficients for each regression line are indicated. Results indicate that parenchymal decreases in levels of native αII-spectrin (280 kDa) are associated with increases in CSF accumulation while increased parenchymal levels of calpain-mediated αII-SBDPs (150 and 145 kDa) are associated with increased CSF accumulation. On average, there were no changes in parenchymal or CSF levels of the caspase-3-mediated 120 kDa αII-SBDP, although individual rats at different time points showed some increase in CSF levels of the 120 kDa product.

when CSF from TBI animals was stored in individual aliquots at either −85°C or at ambient laboratory temperature (~26°C) without protease inhibitors for 48 h, αII-SBDP levels from ambient temperature aliquots were only decreased by 28% compared to aliquots stored at −85°C (Fig. 5). Importantly, the relative stability of αII-SBDP protein in CSF at ambient temperature further indicates this protein as a useful biomarker after TBI.

For cortical and CSF levels of calpain-cleaved 145 kDa αII-SBDP, both slopes of the regression lines were steep and positive indicating large increases in the 145 kDa αII-SBDP in brain and CSF over days (Fig. 4). ANOVA indicated no significant difference ($F = 0.69$, $p = 0.4153$) between slopes indicating that the relative accumulation of 145 kDa αII-SBDP in cortex and CSF were similar as compared to the respective controls. Comparison of slopes for 150 kDa and 145 kDa αII-SBDPs in the brain revealed that the slope of the brain 145 kDa αII-SBDP over days was considerably steeper than the slope of the brain 150 kDa αII-SBDP. This result indicates that brain 145 kDa αII-SBDP protein levels accumulate at a greater rate over days than brain 150 kDa αII-SBDP protein levels. This observation is most likely the result of lower basal levels of brain 145 kDa αII-SBDP than brain 150 kDa αII-SBDP in sham-injured animals and of continued calpain digestion of the larger 150 kDa αII-SBDP to the smaller 145 kDa αII-SBDP over time.

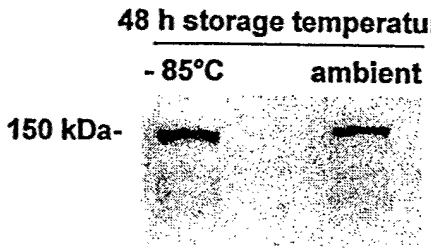

Fig. 5 Stability of αII-SBDP in CSF after prolonged storage in the absence of protease inhibitors at ambient laboratory temperature. The αII-SBDP protein levels only decreased by 28% when stored at ambient temperature (~26°C) for 48 h compared to identical samples stored at −85°C for 48 h. These results indicate that αII-SBDPs in CSF are relatively stable at room temperature. This is an important practical consideration for clinical utility.

For cortical and CSF levels of caspase-3-cleaved 120 kDa αII-SBDP, both slopes were nearly horizontal, indicating no increased accumulation of caspase-3-generated 120 kDa αII-SBDP over days after TBI (Fig. 4). In addition, ANOVA indicated no significant difference between slopes ($F = 0.002$, $p = 0.9621$).

Erythroid αI-spectrin versus non-erythroid αII-spectrin

After head injury, the most likely source of CSF contamination will be from blood. Both neurons and blood

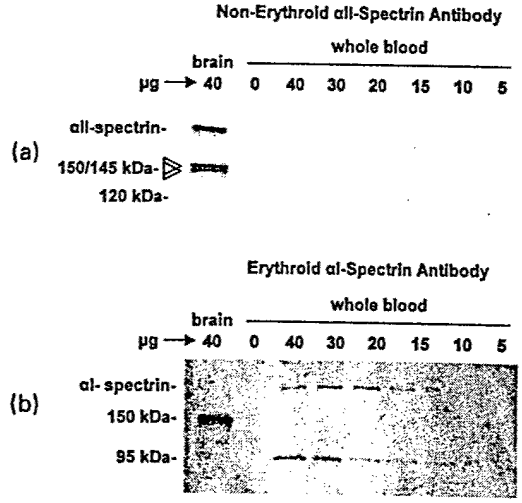

Fig. 6 (a) Non-erythroid αII-spectrin protein is not detected in whole blood. After TBI, the most probable source of non-CNS accumulation of proteins in the CSF is from blood. This immunoblot demonstrates that non-erythroid αII-spectrin is detectable in brain protein homogenates but not in blood protein homogenates. (b) In contrast, use of an erythroid αI-spectrin antibody on the same blot that has been stripped and re-probed reveals immunoreactivity for both blood and brain spectrin. These results demonstrate that potential blood contamination of CSF samples does not affect detection of brain-derived αII-spectrin.

contain the erythroid form αI-spectrin protein. However, erythrocytes do not contain non-erythroid αII-spectrin protein. To demonstrate that the source of αII-spectrin immunoreactivity in the CSF is not blood borne, we probed immunoblots containing various concentrations of whole blood proteins and brain protein with either an erythroid anti-αI-spectrin antibody or with an anti-αII-spectrin antibody (Fig. 6a,b). As predicted, no immunoreactivity was observed at any concentration of whole blood protein (0–40 μg) while brain spectrin was highly reactive to the non-erythroid anti-αII-spectrin antibody. In contrast, both the brain and blood protein samples were immunoreactive to the erythroid anti-αI-spectrin antibody. This result clearly indicates that use of the non-erythroid, but not the erythroid, anti-spectrin antibody can be used to discriminate non-blood borne spectrin protein in CSF samples.

Discussion

This paper provides the first evidence for accumulation of non-erythroid αII-spectrin protein and calpain-mediated αII-SBDPs in CSF after TBI. Detection of calpain-specific proteolytic fragments to αII-spectrin were confirmed with two antibodies, one that recognizes both intact αII-spectrin and calpain-specific SBDPs (FG 6090 Ab), and one that recognizes only the N-terminal region of calpain-cleaved 150 kDa SBDP (SBDP150 Ab). Results of this investigation indicate that CSF detection of αII-spectrin and αII-SBDPs can provide both a sensitive surrogate biochemical measure of TBI pathology and provide important information about specific neurochemical events that have occurred in the brain after TBI. To our knowledge, this is the first investigation of any CNS pathology to indicate that identification of accumulated CSF proteins or protein metabolic products can be used to infer specific neurochemical events (i.e. calpain activation) in the brain. Thus, use of αII-SBDPs as surrogate biochemical markers of TBI has important clinical ramifications for assessment of outcome after injury and for determination of specific pathological proteolytic cascades known to occur after TBI. Although other CNS proteins have been detected in CSF after brain injury (e.g. S-100B) and have been correlated with outcome, these proteins offer no insight into pathological mechanisms that have occurred in the brain after TBI. Obviously, identification of metabolic products with known neurochemical etiology will be beneficial for appropriate application of targeted therapeutics (such as calpain inhibitors) after TBI.

Calpain and caspase-3 cysteine proteases are important mediators of cell death and dysfunction in numerous CNS diseases and injuries including TBI. The calpains have historically been associated with necrotic (oncotic) cell death although recent evidence indicates a role in apoptotic cell death as well (Linnik *et al.* 1996; Nath *et al.* (1996a;) Q5 change to 'a, b' Newcomb *et al.* 1998; Pike *et al.* 1998b). Numerous investigations have reported calpain activation after TBI (Saatman *et al.* 1996a, 2000; Kampfl *et al.* 1997; Newcomb *et al.* 1997; Posmantur *et al.* 1997; Pike *et al.* 1998a) and inhibitors of calpains have been shown to confer neuroprotection after TBI (Posmantur *et al.* 1997; Saatman *et al.* 1996b, 2000). Caspase-3 is a critical executioner of apoptosis and caspase-3 activation has been reported in *in vitro* (Shah *et al.* 1997; Allen *et al.* 1999; Pike *et al.* 2000b) and *in vivo* (Beer *et al.* 2000; Yakovlev *et al.* 1997; Pike *et al.* 1998a; Clark *et al.* (2000a)) models of TBI. Q6 Change to '2000b' However, it should be noted that at least in our hands, the magnitude of calpain activation after TBI is much greater than that of caspase-3, and that at the moderate level of brain injury employed in the current study, caspase-3 is only transiently elevated in deep, non-cortical brain regions (Pike *et al.* 1998a). This result most likely accounts for the detection of relatively minimal amounts of the 120 kDa caspase-3-mediated αII-SBDP in CSF after TBI. In contrast to our injury model, Beer *et al.* (2000) have observed prominent levels of caspase-3 activation in the cortex after cortical impact TBI. However, while our cortical impact model is typically characterized by prominent tissue necrosis and progressive cortical cavitation to the gray–white interface (Kampfl *et al.* 1996; Newcomb *et al.* 1997;

Dixon *et al.* 1998; Newcomb *et al.* 1999; Pike *et al.* 2000a), the model employed by Beer *et al.* (2000) was not. Thus, differences in injury magnitude may be important factors affecting calpain and/or caspase-3 activation after TBI, and this hypothesis warrants further investigation. However, it should be pointed out that although caspase-3 activation has not been a prominent feature in our model of cortical impact TBI, we have detected substantial levels of apoptotic cell death in the cortex after TBI (Newcomb *et al.* 1999). This apparent discrepancy between apoptotic cell death and caspase-3 activation raises the intriguing possibility that apoptosis may occur via a caspase-3-independent pathway after TBI. This observation also warrant further examination.

That different injury magnitudes may result in differential activation of calpain or caspase-3 proteases has important implications for targeted therapeutic intervention after TBI, and importantly, further validates the utility of using surrogate markers of TBI that have known neurochemical etiologies. For example, the current investigation detected CSF accumulation of the calpain-mediated αII-SBDP and not the caspase-3-mediated αII-SBDP. Based on this evidence, administration of calpain but not caspase-3 inhibitors would be predicted to have the most beneficial effect on outcome. However, other injury magnitudes may result in more caspase-3 activation indicating use of caspase-3 inhibitors or a combination of calpain/caspase-3 protease inhibitors. Thus, surrogate measures of TBI will result in selective pharmaceutical therapies based on clinical assessment of neuropathology, and this approach is a superior strategy to promiscuous prophylactic administration of unnecessary and potentially harmful compounds.

The most probable source of peripheral contamination of the CSF after TBI will be blood born. Indeed, we did detect visible red blood cell contamination of CSF after experimental TBI (which was removed by centrifugation). However, our control experiments with brain and whole blood immnuoblots (Fig. 6a,b) clearly demonstrated that the non-erythroid anti-αII-spectrin antibody did not detect any αII-spectrin protein in whole blood samples. Conversly, the erythroid αI-spectrin antibody labeled both brain and blood samples. These results indicate that the major source of potential peripheral CSF contamination after TBI, blood, is not detected by the non-erythroid anti-αII-spectrin antibody. This finding supports the utility of αII-spectrin and αII-SBDPs as surrogate biomarkers of injury after TBI, and importantly, as biomarkers of calpain and/or caspase-3 activation after TBI.

One caveat to the current investigation is the finding that there was more variability in levels of CSF SBDPs than there were in brain levels of SBDPs. This variability is indicated by the larger error bars in Fig. 2 and 4 and can be observed in individual animals in Fig. 1. The reason for the larger variability in CSF protein accumulation is unknown, but may reflect differences in individual animal's CSF circulation after TBI. For example, differences in increased intracranial pressure after TBI may restrict passage of CSF through various foramina that may preclude detection of secreted proteins into the cisterna magna (source of CSF in the present study). Additional studies should examine differences in intraventricular versus intracisternal levels of accumulated SBDPs.

Nonetheless, future studies focused on development of neuron-specific antibodies targeted against calpain-specific and caspase-3-specific αII-SBDPs (such as the SBDP150 Ab) will further strengthen the utility and specificity of αII-SBDPs as surrogate markers of brain injury. In addition, development of enzyme-linked immunosorbent assays (ELISA) will allow greater quantification of calpain and caspase-3 SBDPs and provide a more rapid and practical approach to CSF detection of these proteins.

Acknowledgements

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Temporal and spatial profile of caspase 8 expression and proteolysis after experimental traumatic brain injury

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Abstract

Recent studies have demonstrated that the downstream caspases, such as caspase 3, act as executors of the apoptotic cascade after traumatic brain injury (TBI) *in vivo*. However, little is known about the involvement of caspases in the initiation phase of apoptosis, and the interaction between these initiator caspases (e.g. caspase 8) and executor caspases after experimental brain injuries *in vitro* and *in vivo*. This study investigated the temporal expression and cell subtype distribution of procaspase 8 and cleaved caspase 8 p20 from 1 h to 14 days after cortical impact-induced TBI in rats. Caspase 8 messenger RNA levels, estimated by semi-quantitaive RT-PCR, were elevated from 1 h to 72 h in the traumatized cortex. Western blotting revealed increased immunoreactivity for procaspase 8 and the proteolytically active subunit of caspase 8, p20, in the ipsilateral cortex from 6 to 72 h after injury, with a peak at 24 h after TBI. Similar to our previous studies, immunoreactivity for the p18 fragment of activated caspase 3 also increased in the current study from 6 to 72 h after TBI, but peaked at a later timepoint (48 h) as compared with proteolyzed caspase 8 p20. Immunohistologic examinations revealed increased expression of caspase 8 in neurons, astrocytes and oligodendrocytes. Assessment of DNA damage using TUNEL identified caspase 8- and caspase 3-immunopositive cells with apoptotic-like morphology in the cortex ipsilateral to the injury site, and immunohistochemical investigations of caspase 8 and activated caspase 3 revealed expression of both proteases in cortical layers 2–5 after TBI. Quantitative analysis revealed that the number of caspase 8 positive cells exceeds the number of caspase 3 expressing cells up to 24 h after impact injury. In contrast, no evidence of caspase 8 and caspase 3 activation was seen in the ipsilateral hippocampus, contralateral cortex and hippocampus up to 14 days after the impact. Our results provide the first evidence of caspase 8 activation after experimental TBI and suggest that this may occur in neurons, astrocytes and oligodendrocytes. Our findings also suggest a contributory role of caspase 8 activation to caspase 3 mediated apoptotic cell death after experimental TBI *in vivo*.

Keywords: apoptosis, astrocyte, caspase 8, neuron, oligodendrocyte, traumatic brain injury.

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Traumatically evoked brain injury is a major cause of morbidity and mortality (Thurman *et al.* 1999). Studies over the last two decades have demonstrated that a significant amount of CNS damage after traumatic brain injury (TBI) occurs as a result of secondary autodestructive insults (Hayes *et al.* 1992; Faden 1996; McIntosh *et al.* 1998). Secondary injury involves a complex cascade of biochemical events that contributes to delayed tissue damage and cell death (Kermer *et al.* 1999; Graham *et al.* 2000). Importantly, recent research reported on a potential role for apoptosis in

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Abbreviations used: CNPase, 2′,3′-cyclic-nucleotide-3′-phosphodiesterase; FCS, fetal calf serum; GFAP, glial fibrillary acidic protein; NeuN, neuron specific nuclear protein (neuronal nuclei); PBS, phosphate buffered saline; TBI, traumatic brain injury; TBS, Tris-buffered saline; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine-biotin nick-end labeling.

cell degeneration after cerebral and spinal cord ischemia (Nitatori *et al.* 1995; Kato *et al.* 1997; Charriaut-Marlangue *et al.* 1998), traumatic spinal cord injury (Crowe *et al.* 1997; Liu *et al.* 1997), and TBI *in vitro* (Shah *et al.* 1997; Pike *et al.* 2000) and *in vivo* (Rink *et al.* 1995; Conti *et al.* 1998; Newcomb *et al.* 1999; Beer *et al.* 2000b).

Although a potential role for apoptosis in neuronal and glial cell damage after TBI has been suggested, little is known about the molecular mechanisms involved. However, recent evidence implicates a distinct class of proteases, referred to as caspases. So far, 14 mammalian caspases have been described (Nicholson 1999). Based on their proteolytic specifities, caspases further divide into three groups: the inflammatory caspases (e.g. caspase 1), which mediate cytokine maturation (Cerretti *et al.* 1992); the caspases involved in apoptotic cell death, which segregate into initiator enzymes, such as caspase 8 and caspase 9; executioner caspases, such as caspase 3 (Cohen 1997; Cryns and Yuan 1998). Caspases are synthesized as inactive pro-enzymes that contain three domains (Nicholson 1999), an N-terminal prodomain (approximately 3–24 kDa), a large subunit (approximately 17–21 kDa) and a small subunit (approximately 10–13 kDa). Depending on the cell type, procaspases have been shown to reside in various subcellular localizations (Qin *et al.* 2001; Shikama 2001) and are activated through proteolytic processing and association of the large and small subunits to form a catalytic heterotetramer (Walker *et al.* 1994).

Activation of the executioner caspase 3 has been shown in numerous chronic and acute disorders of the nervous system. For example, caspase 3 processing has been demonstrated in Alzheimer's (Stadelmann *et al.* 1999; Khan *et al.* 2000) and Parkinson's disease (Mogi *et al.* 2000). Further, caspase 3 mediated neuronal and glial cell degeneration has been found in experimental models of cerebral and spinal cord ischemia (Hayashi *et al.* 1998; Namura *et al.* 1998) and spinal cord injury (Springer *et al.* 1999). Importantly, recent data have also suggested a contributory role for activated caspase 3 in apoptotic degeneration of neurons, astrocytes and oligodendrocytes after TBI *in vivo* (Yakovlev *et al.* 1997; Beer *et al.* 2000b; Clark *et al.* 2000).

Current evidence also indicates that in receptor-triggered apoptosis the main pathway for caspase 3 activation is direct activation by caspase 8 (Scaffidi *et al.* 1998; Stennicke *et al.* 1998). Importantly, recent data suggest that receptor-mediated apoptosis indeed occurs in acute CNS injuries (Ertel *et al.* 1997; Felderhoff-Mueser *et al.* 2000). For example, increased expression of Fas and caspase 8 has been shown after experimental spinal cord ischemia (Matsushita *et al.* 2000). In addition, increased Fas and Fas ligand immunoreactivity (Beer *et al.* 2000a) and caspase 3 activation have been reported following TBI in the rat (Beer *et al.* 2000b; Clark *et al.* 2000), suggesting a putative link between the activation of caspase 8 and caspase 3 after TBI *in vivo*. However, to our knowledge no study to date has concurrently investigated changes in the expression and activity of both caspase 8 and caspase 3 in trauma-induced CNS degeneration.

To further investigate potential changes of caspase 8 and caspase 3 expression after experimental TBI, rodents were subjected to a widely used model of experimental brain injury: lateral cortical impact injury (Dixon *et al.* 1991; Franz *et al.* 1999; Beer *et al.* 2000a,b). The present study employed semiquantitative RT-PCR and western blot analyses of procaspase 8, cleaved caspase 8 p20, and processed caspase 3 to determine the relative temporal profile of caspase 8 to caspase 3 expression and activation from 1 h to 14 days after experimental TBI. Immunohistochemical examinations were performed to investigate the cell subtype distribution of caspase 8 after impact injury *in vivo*. Further, TUNEL was used to assess whether caspase 8 and caspase 3 immunopositive cells exhibit morphological features of DNA damage consistent with apoptotic phenotype after TBI in the rat.

Materials and methods

Rat model of traumatic brain injury

A controlled cortical impact device was used to induce a moderate level of TBI, as previously described (Dixon *et al.* 1991; Franz *et al.* 1999). In brief, adult male Sprague–Dawley rats (250–350 g) were intubated and anesthesized with 2% halothane in a 2 : 1 mixture of N_2O/O_2. Core body temperature was monitored continuously using a rectal thermistor probe and maintained at 36.5–37.5°C by a heating pad. Animals were mounted in a stereotaxic frame on the injury device in a prone position secured by ear and incisor bars. A midline incision was made, the soft tissues were reflected, and two 7-mm craniotomies were made adjacent to the central suture, midway between lambda and bregma. The dura was kept intact over the cortex. Injury was induced by impacting the right (ipsilateral) cortex with a 6-mm diameter aluminum tip at a rate of 4 m/s. The injury device was set to produce a tissue deformation of 2 mm. Impact velocity was measured directly by a linear variable differential transformer (Shaevitz Model 500 HR; Shaevitz, Detroit, MI, USA), which produces an analog signal that was recorded by a PC-based data acquisition system for analysis of time/displacement parameters of the impactor. This magnitude of injury has previously been associated with significant cell degeneration restricted to the contusion site (Franz *et al.* 1999; Beer *et al.* 2000a,b). After trauma, animals were extubated and immediately assessed for recovery of reflexes (Dixon *et al.* 1991). Sham-injured animals underwent identical surgical procedures but did not receive impact injury. Naive animals were not exposed to any injury-related surgical procedures. Ninety animals were used in this study (naive rats, $n = 10$; sham-injured rats, $n = 12$; injured rats, $n = 68$). Animal care and experimental protocols complied with the guidelines outlined in the *Guide for the Care and Use of Laboratory Animals*, Austrian Department of Health and Science, and were approved by the University of Innsbruck Medical School Animal

Table 1 Systemic parameters

	Prior to craniotomy ($n = 20$)	Post surgery	
		sham ($n = 4$)	injured ($n = 16$)
MABP (mmHg)	101 ± 5	97 ± 8	103 ± 6
pH	7.46 ± 0.01	7.43 ± 0.02	7.44 ± 0.02
PaO_2 (mmHg)	142 ± 4	79 ± 12	85 ± 9
$PaCO_2$ (mmHg)	43 ± 6	41 ± 3	42 ± 5
Rectal temperature (°C)	37.1 ± 0.2	37.3 ± 0.2	36.9 ± 0.1

Values are mean ± SD; MABP, mean arterial blood pressure.

Welfare Committee. Importantly, all efforts were made to minimize animal suffering and to reduce the number of animals used.

Assessment of physiologic parameters

In a subgroup of animals (sham-injured rats, $n = 4$; injured rats, $n = 16$) systemic parameters were monitored as described by Dixon *et al.* (1991). Briefly, a 22-gauge Teflon catheter was advanced into the abdominal aorta through a left femoral arteriotomy for arterial blood pressure measurement and arterial blood sampling. Blood samples (100 μL) were analyzed for pH, arterial oxygen pressure (PaO_2), and arterial pressure of carbon dioxide ($PaCO_2$), (Table 1) using an AVL Omni 4 (Diamond Diagnostics, Holliston, MA, USA) blood gas analyzer before craniotomy and 5 min after surgery. All parameters were within the normal physiological range (Table 1) (Krinke 2000).

Sample preparation

All animals were given a lethal dose of phenobarbital intraperitoneally (20 mg/kg; Tyrol Pharma, Kundl, Austria) and subsequently killed by decapitation 6 h, 24 h, 48 h, 72 h, 7 days and 14 days after TBI ($n = 4$ for each time after injury, $n = 4$ for naive and sham-injured animals). Both cortices and hippocampi (ipsilateral and contralateral to the injury site) were removed. Excision of both cortices beneath the craniotomies extended ~4-mm laterally, ~7-mm rostrocaudally, and to a depth extending to the white matter. All samples were immediately frozen in liquid nitrogen. The microdissected tissue was homogenized at 4°C in ice-cold homogenization buffer containing 20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 7.1), 2 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.3 mM phenylmethylsulfonylfluoride (PMSF), and 0.1 mM leupeptin. Chelators and protease inhibitors (Sigma, St Louis, MO, USA) were added to prevent endogenous *in vitro* activation of proteases and subsequent artifactual degradation of caspase 8 and caspase 3 during tissue processing.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis, immunoblotting and quantification

Protein concentrations were determined by bicinchoninic acid microprotein assay (Sigma) with albumin standards. Protein-balanced samples were prepared for polyacrylamide gel electrophoresis in two-fold loading buffer containing 0.25 M Tris (pH 6.8), 0.2 M dithiothreitol, 2% sodium dodecyl sulfate, 0.005% bromophenol blue and 5% glycerol in distilled water. Samples were heated for 5 min at 95°C. Sixty micrograms of protein per lane was routinely resolved on 16% Tris/glycine gels (Invitrogen, Groningen, the Netherlands). After separation, proteins were transferred to nitrocellulose membranes using western blotting with transfer buffer made up of 0.192 M glycine and 0.025 M Tris (pH 8.3). Coomassie blue (Bio-Rad, Hercules, CA, USA) and Ponceau red (Sigma) stainings were performed to confirm that equal amounts of protein were loaded in each lane. Five percent non-fat milk in phosphate buffered saline (PBS) with 0.05% Tween 20 was used to reduce non-specific binding. Immunoblots were probed with either a mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), reacting with the p20 subunit and precursor of caspase 8, diluted 1 : 1000, or a rabbit polyclonal antiserum (CM1; IDUN Pharmaceuticals, La Jolla, CA, USA; dilution 1 : 5000), directed against the p18 subunit of activated caspase 3. Specifity and sensitivity of CM1 has been described in detail in previous investigations (Namura *et al.* 1998; Srinivasan *et al.* 1998; Beer *et al.* 2000b). After incubation with primary antibodies overnight at 4°C, nitrocellulose membranes (Amersham Pharmacia Biotech, Uppsala, Sweden) were incubated with secondary antibodies linked to horseradish peroxidase (Amersham Pharmacia Biotech) for 1 h at 20°C (automated climate control). Enhanced chemiluminescence reagents (Amersham Pharmacia Biotech) were used to visualize the immunolabeling on X-ray film. In each blot, the constitutively expressed protein α-tubulin (Sigma) was used as an internal standard to further indicate that sample processing was carried out correctly.

Semiquantitative RT-PCR

Total RNA was isolated from frozen ipsilateral and contralateral cortex and hippocampus of naive ($n = 2$), sham-injured ($n = 4$), and injured animals (1 h, 6 h, 24 h, 48 h and 72 h; $n = 4$ for each time point after injury) with Trizol reagent (Life Technologies, Rockville, MD, USA). Ten micrograms of total RNA was treated with 1 U of amplification grade DNase I (Life Technologies) to eliminate residual genomic DNA and was reverse transcribed into first-strand cDNA using Superscript II reverse transcriptase (Life Technologies) with oligo(dT) as primer. The resulting cDNAs were diluted to 100 μL and subjected to PCR analysis. Each PCR mixture contained equal amounts of diluted cDNA corresponding to 200 ng of total RNA, 100 pM of each primer, 10 pM dNTPs, onefold Ampli-Taq reaction buffer and 2.5 U Ampli-Taq-Gold DNA polymerase (PE Biosystems, Foster City, CA, USA). All cDNAs were amplified with primers specific for the housekeeping

gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH; GenBank Acc. No. X02231; 5′-CCCACGGCAAGTTCAACGG-3′ and 5′-CTTTCCAGAGGGGCCATCCA), and caspase 8 (GenBank Acc. No. AF279308; 5′-ACTGGCTGCCCTCAAGTTCCTGTGC-3′ and 5′-TCCCTCACCATTTCCTCTGGGCTGC-3′). PCR amplification was carried out for 34 cycles of 45 s at 94°C, 45 s at 60°C, and 45 s at 72°C, followed by a final step of 10 min at 72°C in the UNO II Thermocycler (Biometra, Göttingen, Germany). The number of cycles and reaction temperature conditions were optimized to provide a linear relationship between the quantity of input template and the quantity of PCR product. PCR products were analyzed by agarose gel electrophoresis in 2% NuSieve agarose gels (FMC BioProducts, Rockland, ME, USA) and visualized by ethidium-bromide staining. The identity of the PCR products obtained was confirmed by Southern blot analysis using an internal oligonucleotide as hybridization probe.

Immunohistochemistry

Prior to perfusion, animals from all treatment groups were given a lethal injection of phenobarbital (20 mg/kg intraperitoneally). Rats were transcardially perfused through the left ventricle (120 mL of 0.9% saline and 200 mL of 4% paraformaldehyde) at 6 h, 24 h, 48 h, 72 h, 7 days and 14 days after TBI ($n = 4$ for each time point after injury; $n = 4$ for sham-injured and naive animals). The brains were removed, grossly sectioned coronally at 2-mm intervals, processed through graded alcohols and xylene substitute (Histoclear; National Diagnostics, Atlanta, GA, USA), and routinely embedded in paraffin. Sections were cut at 3–4 μm on a rotary microtome, mounted on aminoalkylsilated glass slides, and processed for immunohistochemistry as follows: deparaffinized and rehydrated sections were microwaved in 10 mM sodium citrate buffer, pH 6.0, and allowed to cool to room temperature. Endogenous peroxidase was blocked by treatment with 0.3% H_2O_2 in methanol followed by incubation with 10% fetal calf serum (FCS) in Tris-buffered saline (TBS) for 60 min. Rabbit polyclonal antibodies against caspase 8 (The Burnham Institute, La Jolla, CA, USA) and caspase 3 p18 (IDUN Pharmaceuticals) were diluted 1 : 5000 in 10% FCS and permitted to bind overnight at 4°C.

Rabbit antiserum against caspase 8 was generated as previously described (Krajewska *et al.* 1997) using recombinant catalytic C-terminal fragment of human caspase 8 protein using construct pET15b MGS H_6-Ser216-TAA. This protein was expressed in BL 21 (DE3) cells by induction with 1 mM IPTG. After cell growth and lysis, the clarified cell lysate was applied to an Ni-NTA column and eluted with an imidazole gradient. The pooled caspase 8 fractions were dialyzed against 50 mM Tris at pH 8.8 and applied to a FPLC Mono Q HR 10/10 column (Amersham Pharmacia Biotech) and eluted with an NaCl gradient. New Zealand white female rabbits were injected subcutaneously with a mixture of recombinant protein (0.1–0.15 mg protein per immunization) and 0.5 mL Freund's complete adjuvant with the dose divided over 10 injection sites, and then boosted three times at weekly intervals followed by another 3–20 boostings at monthly intervals with 0.15 mg each of recombinant protein immunogen in Freund's incomplete adjuvant. Antiserum specificity was confirmed by pre-absorption with full-length or fully cleaved caspase 8 protein, respectively. This polyclonal antibody reacts with the unprocessed zymogen form of caspase 8 and detects the processed large subunit (p20) of active caspase 8. In addition, specificity of the caspase 8 antiserum has been described recently (Stoka *et al.* 2001).

Biotinylated goat anti-rabbit (Vector Laboratories, Burlingame, CA, USA) was then applied at a dilution of 1 : 200 in 3% rat serum in TBS for 1 h at room temperature followed by avidin-peroxidase (Sigma), diluted 1 : 100 in TBS, also for 1 h at room temperature. The reaction was visualized by treatment with 0.05% 3,3′-diaminobenzidine tetrahydrochloride solution in TBS containing 0.05% H_2O_2. The color reaction was stopped with several washes of TBS. Immunostaining results were confirmed by the use of pre-immune serum from the same animals and by pre-absorption of the polyclonal antibodies with the relevant protein.

For double immunostaining using brightfield chromagens, sections were pretreated with the rabbit polyclonal antiserum against caspase 8 as described above. Sections were then incubated with a mouse anti-neuron-specific nuclear protein (NeuN) antibody (Wolf *et al.* 1996) (Chemicon, Temecula, CA, USA) for neuronal staining. For staining of astrocytes, oligodendrocytes and microglia, a mouse anti-GFAP (Debus *et al.* 1983) (Roche Molecular Biochemicals, Mannheim, Germany), a mouse anti-CNPase (Sprinkle 1989) (Sternberger Monoclonals Inc., Lutherville, MD, USA) and an anti-ED1 monoclonal antibody (Graeber *et al.* 1990) (Serotec, Kidlington, Oxford, UK) were used. All antibodies were diluted 1 : 500 in 10% FCS in TBS and allowed to bind overnight at 4°C. After being rinsed, sections were incubated with a biotinylated horse anti-mouse antibody (Vector Laboratories) at a dilution of 1 : 200 for 1 h at room temperature followed by incubation with an alkaline phosphatase avidin-biotin substrate and then reaction with blue chromagen (Vector Blue; Vector Laboratories). Sections were dehydrated through graded ethanol, cleared in a xylene substitute (Histoclear; National Diagnostics, Atlanta, GA, USA), mounted in Permount (Fisher Scientific, Nepean, Ontario, Canada) and coverslipped. Sections without primary antibodies were similarly processed to control for binding of the secondary antibodies. On control sections no specific immunoreactivity was detected.

Histochemical detection of DNA fragmentation (terminal deoxynucleotidyl transferase-mediated deoxyuridine-biotin nick end labeling)

To confirm the presence of cell degeneration by an apoptotic mechanism, terminal deoxynucleotidyl transferase-mediated deoxyuridine-biotin nick end labeling (TUNEL) was performed as described by Gavrieli *et al.* (1992) with minor modifications. Briefly, for double-label experiments, dewaxed and rehydrated sections of all animal groups from regions between −1.5 and −3.4 mm bregma were stained with primary and secondary antisera as described earlier. Immunohistochemical staining was visualized by exposure to 3-amino-9-ethylcarbazole in *N,N′*-dimethylformamide (Sigma). Sections were then rinsed thoroughly and incubated with labeling mix (TdT buffer containing 100 U/mL TdT and 20 nM/mL biotin-conjugated 16 deoxyuridine) in a humidified chamber for 60 min at 37°C. After three washes in TBS, slides were incubated in Converter alkaline phosphatase for 15 min in a humidified chamber at 37°C. All reagents were purchased from Roche Molecular Biochemicals. The reaction was visualized by treatment for 3 min with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate system (Dako Corporation, Carpinteria, CA, USA). Primary antibody, labeling mix or

secondary antibody were omitted in control sections. Sections were mounted using an aqueous mounting fluid (Dako Corporation) and examined under the light microscope.

Statistical analysis

Semiquantitative evaluation of RT-PCR band density and of immunoreactivity detected by western blotting was performed using computer-assisted two-dimensional densitometric scanning with a MacIntosh computer using the public domain NIH IMAGE program (developed at the US National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/). Relative band densities on RT-PCR and western blots ($n = 1$/blot) were expressed as arbitrary densitometric units for each time point. This procedure was performed for the data of four independent experiments for a total of four different animals per time point. Data acquired in arbitrary densitometric units were transformed to percentages of the densitometric levels observed for scans from sham animals on the same agarose gel (for RT-PCR analysis) and same blot. Group differences were determined by ANOVA and Tukey's *post hoc* honestly significant difference (HSD) test. Values given are means ± SD of four independent experiments. Differences were considered significant when $p \leq 0.05$. For quantitative analysis of immunohistochemistry, the numbers of caspase 8 and caspase 3 positive cells of three non-consecutive sections (each separated by at least 50 μm) of four different animals for each time point were counted by an independent observer in the entire anatomic regions of the cortex from the primary injury zone at bregma − 3.4 mm ± 0.2 mm (Paxinos and Watson 1997) using light microscopy at a magnification of 100×. The total number of caspase 8- and caspase 3-immunopositive cells was obtained for each section. Further, the numbers of cells labeled with anti-caspase 8 antibody and NeuN, GFAP and CNPase were counted on sections (three sections per animal) processed for double-label immunohistochemistry. Values for each animal (four animals per time point) were averaged to calculate the mean number of immunopositive cells per time point (6–72 h after TBI). Cell counts (caspase 8 vs. caspase 3 and double-labeled neurons vs. double-labeled glia) were analyzed with ANOVA and Bonferroni's *post hoc* analysis for selected pairs of columns. Values given are means ± SD of four different animals. Differences were considered significant when $p \leq 0.05$.

Results

Caspase 8 messenger RNA levels increase after TBI

Caspase 8 messenger RNA was detected by semiquantitative RT-PCR analysis in cortical and hippocampal samples (not shown) of sham and injured animals, respectively (Fig. 1). Cortical impact injury resulted in an increase of caspase 8 messenger RNA levels in the ipsilateral cortex (Fig. 1). Starting at 1 h after injury, a significant increase in caspase 8 messenger RNA levels was observed. Rising rapidly, band intensity reached a maximum level by 6 h after the trauma (386% increase relative to sham animals) and remained thereafter at a steady-state level (332% increase relative to sham animals) at 24 h after TBI. Caspase 8 messenger RNA levels then declined thereafter to a level of approximately

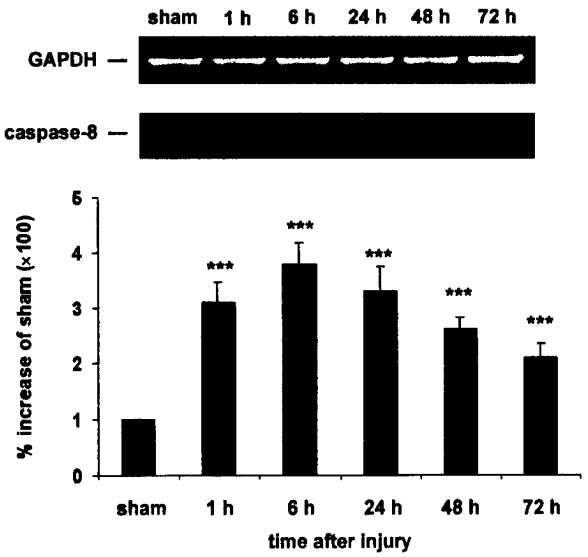

Fig. 1 RT-PCR analysis of caspase 8 mRNA in the ipsilateral cortex following TBI. Cortical samples of single control animals (sham) and single injured animals were prepared for RT-PCR at the indicated times after TBI *in vivo*. Values are presented as percentages of the densitometric levels observed on scans from sham animals visualized on the same agarose gel. Data are mean ± SD values of four independent experiments. Levels of caspase 8 mRNA increased within 1 h after TBI as compared with controls. Levels of caspase 8 mRNA peaked at 6 h after TBI and remained elevated as late as 72 h after the injury. ***$p < 0.001$.

two-fold (220% increase relative to sham animals) above controls at 72 h after the impact. No statistically significant increases in caspase 8 messenger RNA levels were observed in cortical samples contralateral to the injury site and hippocampal samples ipsi- and contralateral to the injury site from 1 h to 72 h after the impact (data not shown).

Proteolytic processing of caspase 8 and caspase 3 occurs after TBI

To determine whether caspase 8 and caspase 3 are activated after TBI, brain extracts from cortex and hippocampus ipsi- and contralateral to the injury site were examined for the expression of the p55 subunit (procaspase 8), the p20 subunit (processed caspase 8) and of the p18 subunit (cleaved caspase 3) by western blotting. Cortical impact injury resulted in an increase of p55 and p20 caspase 8 immunoreactivity in the ipsilateral cortex (Fig. 2a). The p55 and p20 caspase 8 immunoreactivity increased within 6 h after TBI and peaked at 24 h after the impact (376% increase relative to sham animals for p55, and 653% increase as compared with sham animals for p20, respectively), declining thereafter. After 7 and 14 days, no significant increases were evident in the p55 and p20 fragments when compared with levels in sham-injured control animals. Similar to a previous study (Beer *et al.*

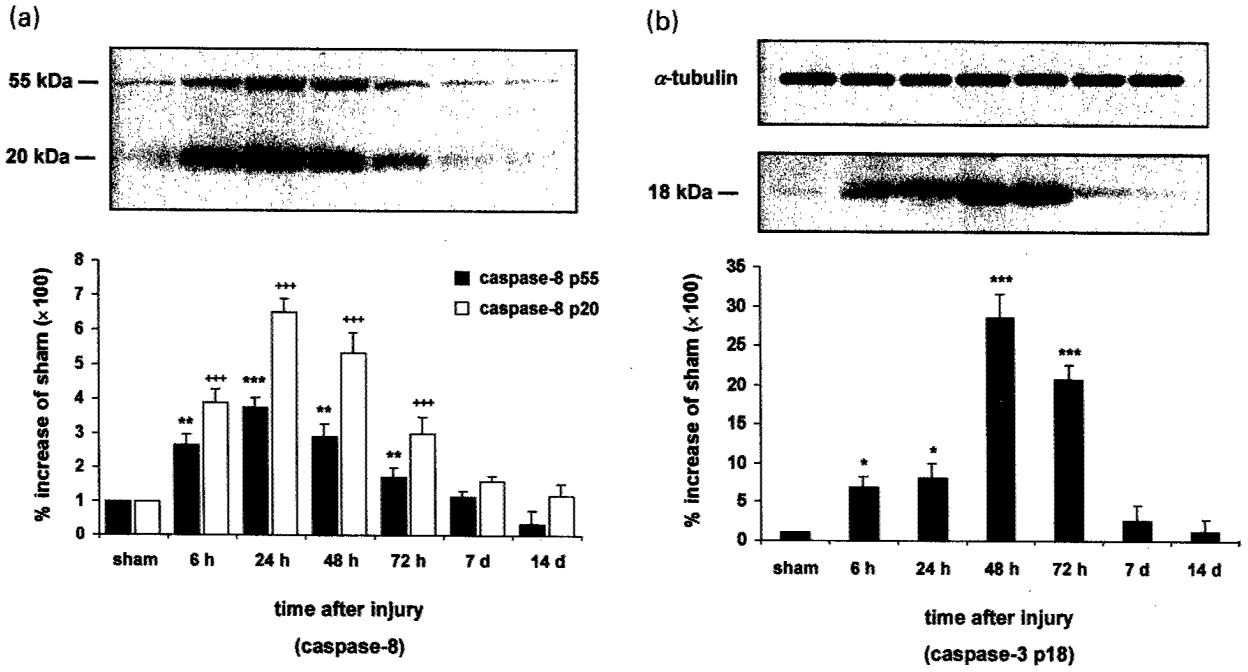

Fig. 2 Time-course of caspase 8 (a) and caspase 3 p18 (b) protein expression after TBI. Samples from single control (sham) and single injured animals were prepared for western blotting between 6 h and 14 days after TBI. Levels of protein are expressed as arbitrary densitometric units. Data were transformed to percentages of the densitometric levels observed on scans from sham animals visualized on the same blot. Values given are mean ± SD of four independent experiments. (a) Ipsilateral cortex: immunoblots demonstrated that procaspase 8 (p55) is constitutively expressed in sham-injured brains. Following TBI, immunoreactivity of p55 (filled bars) (**$p < 0.01$) and p20 (processed caspase 8; open bars) ($^{+++}p < 0.001$) increased significantly at 6 h after TBI and peaked at 24 h post injury. Immunoreactivity of p55 (*$p < 0.05$) and p20 ($^{+++}p < 0.001$) was still significantly increased up to 72 h post trauma. (b) Ipsilateral cortex: the proteolytically active p18 fragment of caspase 3 increased significantly within 6 h after TBI (*$p < 0.05$). p18 immunoreactivity peaked at 48 h after TBI (***$p < 0.001$) and was still significantly elevated at 72 h after impact injury (***$p < 0.001$). α-tubulin was used as an internal standard.

2000b), immunoreactivity for activated caspase 3 (p18) increased within 6 h after TBI in the traumatized cortex (Fig. 2b). However, the maximal increase of p18 immunoreactivity was seen at later times (48 h after TBI; 2850% increase relative to sham animals), when compared with caspase 8 p20. Caspase 3 p18 immunoreactivity then declined to a 2060% increase relative to sham at 72 h after TBI. Similar to proteolyzed caspase 8, no statistically significant differences in caspase 3 p18 immunoreactivity were observed between cortical samples ipsilateral to the injury site at 7 and 14 days after TBI and in cortical samples from sham-injured animals. In addition, no significant increases in p55, p20 and p18 immunoreactivity were seen between sham and injured animals in cortical samples contralateral to the injury site and hippocampal samples ipsi- and contralateral to the injury site between 6 h and 14 days after TBI (data not shown).

Caspase 8 is expressed in traumatized cortical neurons, astrocytes and oligodendrocytes

Ipsilateral and contralateral cortical and hippocampal tissues were examined rostrocaudally from + 0.2 to − 3.8 mm bregma. No caspase 8 immunoreactivity was present in the tissue from sham-injured (Fig. 3a) or naive (data not shown) control rats. Positive immunoreactivity for caspase 8 was found throughout the ipsilateral cortex at the primary injury zone (from − 1.5 to − 3.4 mm bregma) from 6 h to 72 h after the trauma (Figs 3b and c; time point = 24 h after TBI; − 3.4 mm bregma). To further investigate if caspase 8 is expressed in glial and/or neuronal cells, we performed double-labeling experiments for caspase 8 using the neuronal cell specific marker NeuN, the astrocytic marker GFAP, the microglial marker ED-1, and the oligodendroglial marker CNPase. These immunohistochemical analyses of caspase 8-positive cells from 6 to 72 h after TBI (Figs 3i–k; time point = 24 h after trauma; − 3.4 mm bregma) identified labeling with NeuN, GFAP and CNPase, and demonstrated the expression of caspase 8 in cortical neurons (Fig. 3i), astrocytes (Fig. 3j) and oligodendrocytes (Fig. 3k), respectively. Interestingly, immunoreactivity for caspase 8 was observed to be mainly cytosolic in neurons (Figs 3g and i), but appeared rather nuclear in astrocytes (Fig. 3j) and oligodendrocytes (Fig. 3k). No caspase 8 immunoreactivity was detected in microglial cells.

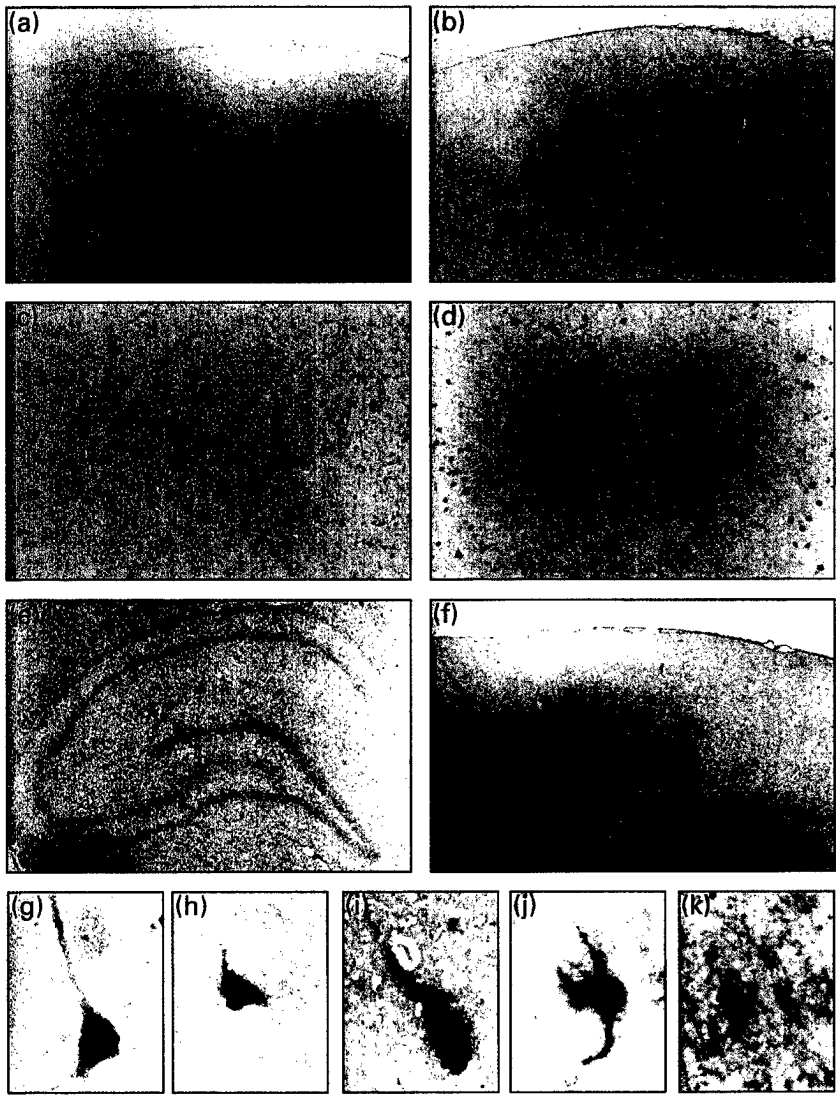

Fig. 3 Cell subtype distribution of caspase 8 in the traumatized cortex (− 3.4 mm bregma) at 24 h after TBI. Sham injured brains showed no specific caspase 8 immunolabeling (a). Low (b), intermediate (c) and high magnification (g) photomicrographs revealed specific caspase 8 expression in the ipsilateral cortex following cortical impact injury. Cells immunopositive for activated caspase 3 are found within similar brain regions (d and h). Double immunostaining experiments with caspase 8 (brown color; i, j and k) and NeuN (blue color; i), GFAP (blue color; j), and CNPase (blue color; k) provided evidence that caspase 8 is expressed in cortical neurons (i), astrocytes (j), and oligodendrocytes (k) after TBI. Magnifications: (a) and (b), 40×; (c) and (d), 100×; (e), 20×; (f), 40×; (g–k), 1000×.

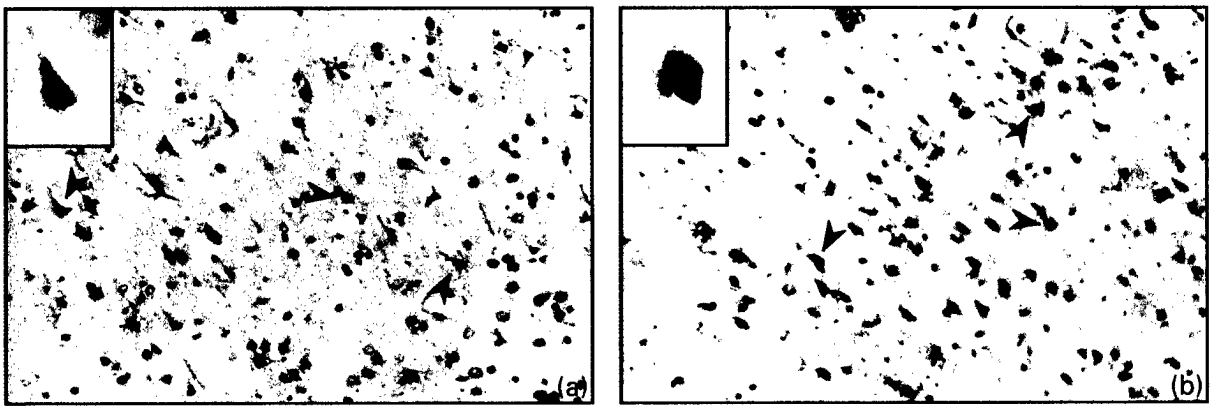

Fig. 4 Appearance of caspase 8 and processed caspase 3 p18 in TUNEL-positive cells. Combined immunohistochemistry for caspase 8 (red color; a) and TUNEL (dark blue color; a) (24 h after TBI) and caspase 3 p18 (red color; b) and TUNEL (dark blue; b) (48 h after TBI) demonstrated caspase 8 (a) and activated caspase 3 (b) in cells with gross nuclear apoptotic-like morphology. TUNEL-positive cells exhibited chromatin condensation and nuclear fragmentation (arrows). Magnifications: (a) and (b), 200×; inserts, 1000×.

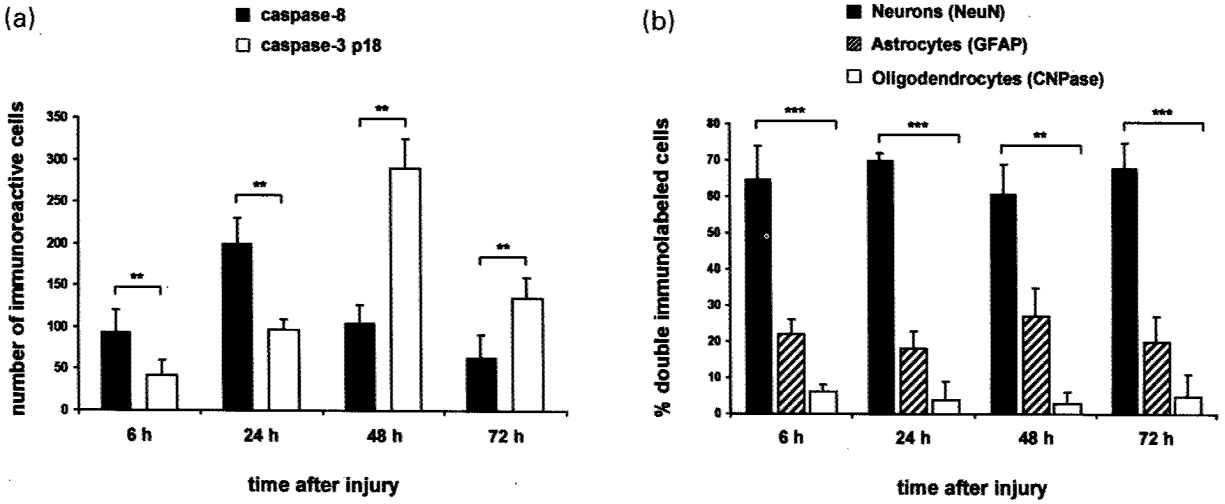

Fig. 5 Quantification of caspase 8 and caspase 3 p18 positive cells in the ipsilateral cortex after TBI (a) and quantification of caspase 8 cell subtype staining (b). Cells were counted in the entire anatomic regions of the cortex at the primary injury zone (bregma −3.4 mm). (a) The number of caspase 8 positive cells (filled bars) was significantly greater than that of caspase 3 p18 positive cells (open bars) before 48 h after TBI. (b) Quantitative analysis was conducted of caspase 8 and NeuN (filled bars), caspase 8 and GFAP (hatched bars), and caspase 8 and CNPase (open bars) immunopositive cells. Columns indicate double-labeled neurons (filled bars), double-labeled astrocytes (hatched bars), and double-labeled oligodendrocytes (open bars) as percentages of caspase 8-positive cells from 6 to 72 h after TBI. Cell counts of caspase 8-positive neurons were significantly higher as compared with caspase 8-positive glia (i.e. astrocytes and oligodendrocytes at 6, 24, 48 and 72 h after TBI, respectively). Cell counts were evaluated by ANOVA with Bonferroni's *post hoc* analysis (**$p < 0.01$, ***$p < 0.001$). Values given are mean ± SD of four different animals per time point.

Caspase 8 immunoreactivity was absent in ipsilateral hippocampal samples (Fig. 3e) and contralateral samples of cortex (Fig. 3f) and hippocampus (data not shown) at all times investigated.

Caspase 8- and caspase 3-immunopositive cells exhibit nuclear apoptotic-like morphology

To verify further an apoptotic component of post-traumatic cell death and to support the possibility that caspase 8 and caspase 3 are associated with trauma-induced apoptosis, sections immunopositive for caspase 8 and caspase 3 p18 were stained with TUNEL to assess DNA damage. Double-labeling experiments demonstrated that a substantial proportion of TUNEL positive cells with shrunken morphology, condensed nuclei and chromatin margination also expressed caspase 8 (Fig. 4a) and activated caspase 3 (Fig. 4b) in layers 2–5 of the injured parietal cortex after TBI. However, cells with either caspase 8 or activated caspase 3 reactivity or gross apoptotic-like morphology alone were also detected.

Quantification of caspase 8- and caspase 3-positive cells and caspase 8 cell subtype staining after TBI

Activated caspase 3 was detected in the ipsilateral traumatized cortex from 6 to 72 h after impact injury. Similar to our findings for caspase 8, p18 positive cells were seen within cortical layers 2–5 at the primary impact zone (Fig. 3d) (Beer *et al.* 2000b). In contrast to caspase 8, the intracellular localization of activated caspase 3 was predominantly nuclear (Fig. 3h). Quantitative analysis of caspase 8- and p18-positive cells revealed that the number of caspase 8- and p18-labeled cells increased up to 24 and 48 h after trauma, respectively, with the number of caspase 8-positive cells being significantly higher until 24 h after TBI (Fig. 5a).

Quantification of caspase 8 and NeuN, GFAP and CNPase revealed that the number of caspase 8-positive neurons is significantly higher as compared with glia (i.e. astrocytes and mature oligodendrocytes) from 6 to 72 h after injury, respectively (Fig. 5b).

Discussion

Our results provide the first evidence for caspase 8 expression and processing after experimental TBI. Proteolyzed caspase 8 appeared in samples of the traumatized cortex from 6 to 72 h after impact injury. Furthermore, double labeling experiments revealed expression of caspase 8 in neurons, astrocytes and oligodendrocytes after experimental brain injury. Moreover, our data indicate that expression of caspase 8 and cleaved caspase 3 p18 is associated with apoptotic-like cell death phenotypes detected in TUNEL-positive cells. Finally, our results suggest that caspase 8 may at least in part contribute to caspase 3-mediated cell death after experimental TBI in the rat.

Reverse transcription PCR and western blotting data revealed increased expression of caspase 8 messenger RNA and increased immunoreactivity for procaspase 8 and caspase 8 p20 in the ipsilateral cortex from 1 h to 72 h after the impact. Similar to our results, current data also suggest that caspase 8 messenger RNA, procaspase 8 and activated caspase 8 are over-expressed at early time points after experimental spinal cord ischemia (Matsushita *et al.* 2000) and focal cerebral ischemia (Harrison *et al.* 2001) in the mouse. Moreover, increased expression of proteolyzed caspase 8 has also been described after experimental focal ischemia in the rat up to 48 h after the insult (Velier *et al.* 1999). In this regard, it is noteworthy that cortical impact injury may produce focal ischemia in the cortex ipsilateral to the injury site (Bryan *et al.* 1995). Thus, reduced cerebral blood flow may have also contributed to activation of caspase 8 in our experiments. Taken together, these data demonstrate that caspase 8 is up-regulated and activated as an early event after various acute CNS injuries *in vivo*.

Previous reports on the cell subtype distribution have found increased immunoreactivity for caspase 8 in neurons after focal ischemia in the rat (Velier *et al.* 1999). In addition, recent data provided evidence that caspase 8 is also over-expressed in neurons following experimental spinal cord ischemia in the mouse (Matsushita *et al.* 2000) and in oligodendrocytes undergoing staurosporine-induced apoptosis *in vitro* (Gu *et al.* 1999). These findings indicate that whereas caspase 8 has been reported only to be weakly expressed in normal brain parenchyma (Velier *et al.* 1999), the brain readily over-expresses caspase 8 after a variety of pathological stimuli. Our immunohistochemical results, however, are the first to show that caspase 8 is over-expressed in neurons, astrocytes and oligodendrocytes after experimental brain injury *in vivo*. Moreover, our findings suggest that caspase 8 expression is mainly cytosolic in neurons, but has a rather nuclear distribution in astrocytes and oligodendrocytes after TBI *in vivo*. In this regard it is noteworthy that caspase 8 can be expressed in both the nuclear and cytosolic compartment (Xerri *et al.* 2000). Therefore, future studies are needed to further investigate the significance of caspase 8 expression in different subcellular compartments of CNS cells after experimental brain injuries *in vivo*.

The exact mechanisms leading to the activation of caspase 8 after CNS injuries have yet not been determined. Recent data suggest that caspase 8 may be activated by receptor-mediated mechanisms including the tumor necrosis factor receptor type-1 (TNF-R1) (Schulze-Osthoff *et al.* 1998) and Fas signaling pathways (Muzio *et al.* 1996; Medema *et al.* 1997). For example, it has been reported that over-expression of α-TNF and Fas ligand may induce apoptosis after experimental cerebral ischemia and brain trauma *in vivo* (Kokaia *et al.* 1998; Shohami *et al.* 1996; Martin-Villalba *et al.* 1999). Moreover, increased Fas expression has been implicated in glutamate-induced apoptotic cell death of CNS neurons *in vitro* (Li *et al.* 1998). Importantly, excessive excitatory amino acid release with subsequent neurotoxicity also has been described after TBI *in vivo* (for review see Globus *et al.* 1995). Finally, recent data by Beer *et al.* (2000a) indicate that the Fas/Fas ligand system is also up-regulated in similar brain regions and at similar times after TBI as caspase 8 expression seen in our study (cortical layers 2–5, and 15 min to 72 h after impact injury, respectively). Taken together, these results suggest that excitatory amino acids and the Fas/Fas ligand system may indeed participate in activation of caspase 8 after TBI. In this regard, it is noteworthy that coexpression of Fas and caspase 8 has been observed in spinal cord neurons after experimental spinal cord ischemia *in vivo* (Matsushita *et al.* 2000).

Recent data suggest that cell loss induced by traumatic spinal cord injury and TBI may be attributed in part to apoptotic mechanisms (for a review see Beattie *et al.* 2000; Raghupathi *et al.* 2000). Moreover, several *in vivo* and *in vitro* studies have documented the significance of caspases in apoptotic cell degeneration following acute CNS injuries (for reviews see Eldadah and Faden 2000; Mattson *et al.* 2000). In addition, it has also been reported that caspase 3 is activated after experimental cerebral ischemia (Namura *et al.* 1998; Velier *et al.* 1999), fluid percussion (Yakovlev *et al.* 1997), and cortical impact models of TBI (Beer *et al.* 2000b; Clark *et al.* 2000). Our western blotting data also indicate that caspase 3 is activated after TBI, and our double-labeling immunohistochemical studies using TUNEL and p18 antiserum demonstrated caspase 3-positive cells with gross DNA damage in the traumatized cortex, suggesting a mechanistic link between caspase 3 activation and apoptosis.

Recent evidence from *in vitro* studies suggests that caspase 3 can be activated directly by caspase 8 (Stennicke *et al.* 1998). However, coexpression of caspase 8 and activated caspase 3 after various CNS injuries *in vivo* has not been studied extensively. One recent report indicated that caspase 8 and activated caspase 3 are coexpressed within CNS cells after experimental spinal cord ischemia up to 24 h after the insult (Matsushita *et al.* 2000). Our present findings provide further evidence of expression of both, caspase 8 and activated caspase 3 in multiple cortical CNS cell populations after TBI *in vivo*. Importantly, the expression of caspase 8 and activated caspase 3 in similar cortical brain regions suggests that caspase 8 may participate in caspase 3 activation in cortical CNS cells after TBI. Moreover, the greater number of caspase 8 positive cells (compared with caspase 3 immunoreactive cells) at early time points (6 and 24 h post trauma) followed by a greater number of caspase 3 positive cells (compared with caspase 8 positive cells) at later time points (48 h and 72 h post trauma) supports the hypothesis that caspase 8 may indeed be upstream of caspase 3. However, it was of interest that

caspase 8 immunoreactivity is found also in cells with evidence of DNA damage as indicated by TUNEL. In this regard it is noteworthy that caspase 8 may also function as an amplifying executioner caspase in drug-induced apoptosis *in vitro* (Engels *et al.* 2000). Therefore, future studies have to investigate the exact role of caspase 8 in the activation and/or execution phase of the apoptotic cascade after acute CNS injuries *in vivo*. This also implicates the need for further investigations on the significance of caspase 8 independent activation of caspase 3, including the mitochondrial pathway (Eldadah and Faden 2000).

Our study failed to detect increased expression of caspase 8, activated caspase 3 and apoptotic CNS morphology in the hippocampus ipsilateral to the injury site from 6 h to 14 days after the trauma. This is in contrast to previous reports, which clearly describe features of apoptotic neuronal degeneration in the hippocampus following fluid percussion injury (Yakovlev *et al.* 1997; Conti *et al.* 1998) and cortical impact injury (Clark *et al.* 2000; Colicos and Dash 1996). However, previous studies from our laboratory (Franz *et al.* 1999; Beer *et al.* 2000a,b) have shown that cortical impact injury may not necessarily be associated with hippocampal neuronal degeneration. Probable reasons for discrepancies in the appearance of hippocampal damage may be subtle methodological differences in animal models of TBI. For example, differences in angulation and velocity of the impact devices could account for the presence or absence of hippocampal cell degeneration.

In conclusion, our results provide evidence for induction of caspase 8 expression in cortical neurons and glial cells after TBI *in vivo*. Moreover, our data raise the possibility that caspase 8 may contribute to caspase 3 activation after impact injury in the rat. In addition to these *in vivo* findings, we also provided evidence that caspase 8 and activated caspase 3 may participate in mechanisms of apoptotic CNS cell degeneration in the traumatized cortex. However, future studies are needed to further elucidate the precise role of caspase 8 and activated caspase 3 for cellular CNS degeneration after TBI, including the significance of apoptotic cell death on functional outcome after acute brain injuries *in vivo*.

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